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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

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Use of prion conversion modulating agents

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USE OF PRION CONVERSION MODULATING AGENTS

Field of the invention

This invention relates to the use of apolipoprotein B or apolipoprotein E or fragments thereof for diagnostic, detection, prognostic and therapeutic applications in prion diseases. More specifically, the invention provides the use of apolipoprotein B or apolipoprotein E or fragments thereof for modulating or identifying modulators of the prion protein replication which are implicated in the pathogenesis of transmissible spongiform encephalopathies and other prion diseases.

Background of the invention

Creutzfeldt-Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals are some of the diseases that belong to the group of Transmissible Spongiform Encephalopathies (TSE), also known as prion diseases *(Prusiner, 1991)*. These diseases are characterized by an extremely long incubation period, followed by a brief and invariably fatal clinical disease (*Roos et al., 1973*). To date no therapy is available.

Although these diseases are relatively rare in humans, the risk for the transmissibility of BSE to humans through the food chain has seized the attention of the public health authorities and the scientific community *(Soto at al., 2001)*. Variant CJD (vCJD) is a new disease, which was first described in March 1996 *(Will et al., 1996)*. In contrast to typical cases of sporadic CJD (sCJD), this variant form affects young patients (average age 27 years old) and has a relatively long duration of illness (median 14 months vs. 4.5 months in traditional CJD). A link between vCJD and BSE was first hypothesized because of the association of these two TSEs in place and time *(Bruce, 2000)*. The most recent and powerful evidence comes from studies showing that the transmission characteristics of BSE and vCJD to mice are almost identical and strongly indicating that they are due to the same causative agent *(Bruce et al., 1997)*. Moreover, transgenic mice carrying a human or a bovine gene have now been shown to be susceptible to BSE and vCJD *(Scott et al., 1999)*. Furthermore, no other plausible hypothesis for the occurrence of vCJD has been proposed and intensive CJD surveillance in five European

countries, with a low exposure to the BSE agent, has failed to identify any additional cases. In conclusion, the most likely cause of vCJD is exposure to the BSE agent, probably due to dietary contamination with affected bovine central nervous system tissue.

The nature of the transmissible agent has been matter of passionate controversy. Further research, has indicated that the TSE agent differs significantly from viruses and other conventional agents in that it seems not to contain nucleic acids *(Prusiner, 1998)*. Additionally, the physicochemical procedures that inactivate most viruses, such as disrupting nucleic acids, have proved ineffective in decreasing the infectivity of the TSE pathogen. In contrast, the procedures that degrade protein have been found to inactivate the pathogen *(Prusiner, 1991)*. Accordingly, the theory that proposes that the transmissible agent is neither a virus nor other previously known infectious agent, but rather an unconventional agent consisting only of a protein recently gained widespread acceptability *(Prusiner, 1998)*. This new class of pathogen was called a "prion", short for "proteinaceous infectious particle". In TSE, prions are composed mainly of a misfolded protein named PrP^{Sc} (for scrapie PrP), which is a post-translationally modified version of a normal protein, termed PrP^{C} *(Cohen et al., 1998)*. Chemical differences have not been detected to distinguish these two PrP isoforms and the conversion seems to involve a conformational change whereby the α-helical content of the normal protein diminishes and the amount of β-sheet increases *(Pan et al., 1993)*. The structural changes are followed by alterations in the biochemical properties: PrP^{C} is soluble in non-denaturing detergents, PrP^{Sc} is insoluble; PrP^{C} is readily digested by proteases (also called protease sensitive prion protein) while PrP^{Sc} is partially resistant, resulting in the formation of a N-terminally truncated fragment known as PrPres (protease resistant prion protein) *(Cohen et al., 1998)*.

The notion that endogenous PrP^{C} is involved in the development of infection is supported by experiments in which endogenous PrP gene was knocked out where the animals were both resistant to prion disease and unable to generate new infectious particles *(Bueler et al., 1993)*. In addition, it is clear that during the time between the inoculation with the infectious protein and the appearance of the clinical symptoms, there is a dramatic increase in the amount of PrP^{Sc}.

These findings suggest that endogenous PrP^C is converted to PrP^{Sc} conformation by the action of an infectious form of the PrP molecule *(Soto et al., 2001)*. Prion replication is hypothesized to occur when PrP^{Sc} in the infecting inoculum interacts specifically with host PrP^C, catalyzing its conversion to the pathogenic form of the protein. A physical association between the two isoforms during the infectious process is suggested by the primary sequence specificity in prion transmission *(Telling et al., 1994)* and by the reported *in vitro* generation of PrP^{Sc}-like molecules by mixing purified PrP^C with PrP^{Sc} *(Saborio et al., 2001)*. However, the exact mechanism underlying the conversion is not known.

Investigations with chimeric transgenes showed that PrP^C and PrP^{Sc} are likely to interact within a central domain delimited by codons 96 and 169 *(Prusiner, 1996)* and synthetic PrP peptides spanning the region 109-141 proved to be able to bind to PrP^C and compete with PrP^{Sc} interaction *(Chabry et al., 1998)*.

Based on data with transgenic animals, it has been proposed that additional brain factors present in the host are essential for prion propagation *(Telling et al., 1995)*. It has been demonstrated previously that prion conversion does not occur under experimental conditions where purified PrP^C and PrP^{Sc} are mixed and incubated *(Saborio et al., 1999)* but that the conversion activity is recovered when the bulk of cellular proteins are added back to the sample *(Saborio et al., 1999)*. This finding provides direct evidence that other factors present in the brain are essential to catalyze prion propagation.

The observation that cholesterol depletion decreases the formation of PrP^{Sc} whereas sphingolipid depletion increases PrP^{Sc} formation, suggested that "lipid rafts" (lipid domains in membranes that contain sphingolipids and cholesterol) may be the site of the PrP^c to PrP^{Sc} conversion reaction involving either a raft-associated protein or selected raft lipids *(Fantini et al., 2002)*. However, the role of lipid rafts in prion infectivity is still unclear.

One approach to the treatment and prevention of prion diseases has been to develop agents for blocking the transformation of PrP^c into PrP^{Sc}. Some agents proposed were Congo red dye (US 5,276,059), nerve growth peptides (US 5,134,121), fragments of

prion proteins (US 6,355,610) and beta-sheet breaker peptides (US 5,948,763) but it would be desirable to develop new methods for identifying and inhibiting the prion conversion factor(s).

Apolipoprotein B is the major protein component of the two known atherogenic lipoproteins, Low Density Lipoproteins (LDL) and remnants of triglyceride-rich lipoproteins and is a ligand for the LDL receptor *(Segrest et al., 2001)*. Apolipoprotein E is a constituent of several plasma lipoprotein such as chylomicrons, very low-density lipoproteins (VLDL), and high-density lipoproteins (HDL) (*Lehninger et al., 1993*) and is a ligand for the LDL receptor. Apolipoprotein B and E are known for their prominent role in cholesterol transport and plasma lipoprotein metabolism via LDL receptor interactions. Apolipoprotein E has recently emerged as a major genetic risk factor for Alzheimer's disease, a neurodegenerative disorder.

Summary of the invention

It is an object of the invention to provide a use of peptides or proteins in an assay for the detection of PrP^{Sc} formation in a sample.

It is also an object of the invention to provide a use of peptides or proteins in a screening assay for identifying compounds that modulate the conversion of PrP^{c} into PrP^{Sc}.

It is further an object of the invention to provide a substance which is suitable for the treatment of and/or prevention of and/or delaying the progression of prion related disorders, notably, bovine spongiform encephalopathy (BSE) and Creutzfeld-Jacob Disease (CJD).

In a first aspect, the invention provides a use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof in an assay for the detection of PrP^{Sc} formation in a sample.

In a second aspect, the invention provides a use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and

mimetics thereof in a screening assay for identifying compounds that modulate the conversion of PrP^c into PrP^{Sc}.

In a third aspect, the invention provides a use of a modulator, preferably an inhibitor or an antagonist, of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof for the preparation of a pharmaceutical preparation for the treatment of a prion disease, notably, bovine spongiform encephalopathy (BSE) and a Creutzfeld-Jacob Disease (CJD).

In a fourth aspect, the invention provides a method for the diagnosis or detection of a prion disease within a subject suspected of suffering from such a disease which comprises (i) obtaining a sample from the subject; (ii) contacting a sample from said subject with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof; (iii) contacting the sample obtained from step (ii) with PrP^C or PrP^C contain ing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample.

In a fifth aspect, the invention provides a method of determining a marker that predisposes a subject to a prion disease, comprising (i) obtaining a sample from the subject; (ii) measuring a level of a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment and mimetics thereof in said sample; and (iii) correlating said level of protein obtained in said measuring step with the occurrence of a prion disease.

In a sixth aspect, the invention provides a method for the detection of PrP^{Sc} formation within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof; (ii) contacting the sample obtained from step (i) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

In a seventh aspect, the invention provides a method for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof (a) in the presence of said compound and (b) in the absence of said compound; (ii) contacting the sample obtained from step (i) a and (i) b with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said compound.

In a eighth aspect, the invention provides an assay for the detection of PrP^{Sc} formation within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof; (ii) contacting the sample obtained from step (i) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

In a ninth aspect, the invention provides a screening assay for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof (a) in the presence of said compound and (b) in the absence of said compound; (ii) contacting the sample obtained from step (i) a and (i) b with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said compound.

Detailed description of the invention

The following paragraphs provide definitions of various terms, and are intended to apply uniformly throughout the specification and claims unless an otherwise expressly set out definition provides a different definition.

The term "Gerstmann-Strassler-Scheinker Disease" abbreviated as "GSS" refers to a form of inherited human prion disease. The disease occurs from an autosomal dominant disorder. Family members who inherit the mutant gene succumb to GSS.

The term "prion" shall mean a transmissible particle known to cause a group of such transmissible conformational diseases (spongiform encephalopathies) in humans and animals. The term "prion" is a contraction of the words "protein" and "infection" and the particles are comprised largely if not exclusively of PrP^{Sc} molecules.

"Prions" are distinct from bacteria, viruses and viroids. Known prions include those which infect animals to cause scrapie, a transmissible, degenerative disease of the nervous system of sheep and goats as well as bovine spongiform encephalopathies (BSE) or mad cow disease and feline spongiform encephalopathies of cats. Four prion diseases known to affect humans are Kuru, Creutzfeldt-Jakob Disease (CJD), Gerstmann-Strassler-Scheinker Disease (GSS), and fatal familial insomnia (FFI) *(Prusinier, 1991)*. As used herein prion includes all forms of prions causing all or any of these diseases or others in any animals used -- and in particular in humans and in domestic farm animals.

The term "lipid rafts" refers to small platforms, composed of sphingolipids and cholesterol in the outer exoplasmic layer, connected to Cholesterol in the inner cytoplasmic layer of the bilayer that have been reviewed recently *(Simons et al., 2000)*. Lipid rafts can be isolated as they are insoluble in certain detergents such as triton X-100 at 4°C. Therefore, rafts can be purified as detergent-insoluble membranes (DIMs) or detergent-resistant membranes (DRMs) by ultracentrifugation on sucrose gradients. Rafts are enriched in GPI-anchored proteins, as well as proteins involved in signal transduction and intracellular trafficking. In neurons, lipid rafts act as platforms for the

signal transduction initiated by several classes of neurotrophic factors *(Tsui-Pierchala et al., 2002)*. Example for lipid rafts extraction is given in Example n° 2 §c.

The term "prion conversion factor" refers to a factor comprising proteins, lipids, enzymes or receptors that acts as a co-factor or auxiliary factor involved in the process of conversion of PrP^{C} into PrP^{Sc} and favors the onset and/or progression of the prion disease.

The terms "standardized prion preparation", "prion preparation" and the like are used interchangeably herein to describe a composition containing prions which composition is obtained for example from brain tissue of mammals substantially the same genetic material as relates to PrP proteins, e.g. brain tissue from a set of mammals which exhibit signs or prion disease or for example a composition which is obtained from chronically prion infected cells.

The terms "sensitive to infection", "sensitive to prion infection" and the like are use for a material from a mammal, including cells, that can be infected with an amount and type of prion which would be expected to cause prion disease or symptoms.

By analogy, the terms "resistant to infection", "resistant to prion infection" and the like are used for a material from a mammal, including cells which has the characteristic to be resistant when infected with an amount and type of prion which would be expected to cause prion disease or symptoms and remain uninfected even after several infective prion material inoculations.

The term "sample" refers to a biological extract from a mammal, including cell sample, body fluid, genetic material such as brain homogenate, cells, lipid rafts or purified peptides and proteins.

The term "incubation time" shall mean the time from inoculation of an animal with a prion until the time when the animal first develops detectable symptoms of disease resulting from infection, it also means the time from inoculation of material from a

mammal, e.g. brain homogenate, cells, lipid rafts from cells, with prion until the time when the prion infection is detectable such as through the conversion of PrP^{C} into PrP^{Sc}. Several methods of detection of prion infection and PrP conversion are known by a person skilled in the art.

The term "fraction" refers to any fragment of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example residues of sugars or phosphates, or aggregates of the original polypeptide or peptide. Such molecules can result also from other modifications which do not normally alter primary sequence, for example *in vivo* or *in vitro* chemical derivativization of peptides (acetylation or carboxylation), those made by modifying the pattern of phosphorylation (introduction of phosphotyrosine, phosphoserine, or phosphothreonine residues) or glycosylation (by exposing the peptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes) of a peptide during its synthesis and processing or in further processing steps.

The terms "modulator" or "modulatory compound" refer to molecules that modify the functions and/or properties (such as receptor binding, lipid affinity, enzyme interaction, structural arrangement, synthesis, metabolism) of the natural protein. "Modulators" of "modulatory compounds" include "agonists" and antagonists". Modulators" include peptides, proteins or fragments thereof, peptidomimetics, organic compounds and antibodies.

The term "mimetics" refer to molecules that mimic the functions and/or properties (such as receptor binding, lipid affinity, enzyme interaction, structural arrangement, synthesis, metabolism) of a natural protein. These compounds have for example the property to either enhance a property of the natural protein (i.e. to lead to the same activity when the compound is added to the natural protein as obtained with an increase in concentration in the natural protein) or to exhibit the same property as a natural protein (i.e. to lead to the same activity when the compound replaces the natural protein). "Mimetics" include peptides, proteins or fragments thereof, peptidomimetics and

organic compounds. Examples of apolipoprotein E mimetics are described in US20020128175.

The terms "inhibitor" or "antagonist" refer to molecules that alter partially or impair the functions and/or properties (such as receptor binding, lipid affinity, enzyme interaction, structural arrangement, synthesis, secretion, metabolism) of the natural protein. "Inhibitors" or "antagonists" include peptides, proteins or fragments thereof, peptidomimetics, organic compounds and antibodies. Examples of Apolipoprotein B antibodies are described in *Choi et al., 1997* and in *Wang et al., 2000*. Examples of Apolipoprotein E antibodies are described in *Aizawa et al., 1997* and *Yamada et al., 1997*. Examples of Apolipoprotein antagonists can be antagonists that alter or impair the role of Apolipoproteins B or E in the cholesterol transport pathway. Examples of compounds that alter Apolipoprotein B secretion or synthesis are described in US 6,369,075, US 6,197,972, WO 03002533 and WO 03045921. Other "modulators" or "antagonists" can be modulators of the LDL receptor, preferably LDL-receptor antagonists such as anti-LDL receptor antibodies. Examples of monoclonal antibodies to the LDL receptor are given in WO 0168710.

The term "protein misfolding cyclic amplification assay" or "PMCA assay" is an assay that for the diagnosis or detection of conformational diseases which comprises a cyclic amplification system to increase the levels of the pathogenic conformer such as described for example in WO 0204954.

The term "marker" for a disease refers to a biological parameter or value including a genetic character, inherited protein mutation(s), blood level of a protein or an enzyme that is different from the average value in a heterogeneous population of individuals and whose occurrence correlates with the occurrence of said disease with a statistical significance. A "marker" for a disease or condition is typically defined as a certain cut-off level of a said biological variable. A "marker" provides basis for determining the risk (probability of occurrence) of a disease in a subject.

The term "complex" includes the formation of an entity by the interaction of several molecules, several proteins, several peptides together or with a receptor. These interactions may be reversible and/or transient. These interactions may induce changes in the properties of the interacting molecules, proteins, peptides or receptors.

By "effective amount", it is meant a concentration of peptide(s) that is capable of slowing down or inhibiting the formation of PrP^{Sc} deposits, or of dissolving preformed deposits. Such concentrations can be routinely determined by those of skill in the art. It will also be appreciated by those of skill in the art that the dosage may be dependent on the stability of the administered peptide. A less stable peptide may require administration in multiple doses.

The preparation of antibodies is known by the person skilled in the art. It is referred by "antibody" to a monoclonal antibody, chimeric antibody, humanized antibody, anti-anti-Id antibody or fragment thereof which specifically recognises and binds to Apo B or Apo E and fragments thereof. For example, monoclonal antibodies are obtained though the generation of hybridoma cells lines producing monoclonal antibodies capable of specifically recognising and binding Apo B or Apo E and fragments thereof. More specifically, these monoclonal antibodies are capable of specifically recognising and binding Apo B or Apo E. A monoclonal antibody can be prepared in a conventional manner, e.g. by growing a cloned hybridoma comprising a spleen cell from a mammal immunized with hApo B or hApo E and a homogenic or heterogenic lymphoid cell in liquid medium or mammalian abdomen to allow the hybridoma to produce and accumulate the monoclonal antibody. Preferably, the antibody specifically recognises and binds to Apo B- and/or Apo E-LDL recognizing fragments.

The present invention provides compounds capable of controlling, including increasing and/or inhibiting, the conversion of PrP^C into PrP^{Sc} in prion diseases.
The activity of the compounds of the invention in controlling the conversion of PrP^C into PrP^{Sc} in prion diseases can be detected using, for example, an *in vitro* assay, such as that described by *Saborio et al., 2001* which measures the ability of compounds of the

invention to modulate the conversion of PrP^C into PrP^{Sc}. Results are reported in the Examples.

In an embodiment of the invention, the invention provides a use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof in an assay for the detection of PrP^{Sc} formation in a sample.

In one preferred embodiment of the invention, the peptide or the protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof used in an assay for the detection of PrP^{Sc} formation in a sample binds and/or forms a complex with the LDL receptor.

In another embodiment, the invention provides a use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof in a screening assay for the identifying compounds that modulate the conversion of PrP^c into PrP^{Sc}.

In another preferred embodiment of the invention, the peptide or the protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof used in a screening assay for the identifying compounds that modulate the conversion of PrP^c into PrP^{Sc} binds and/or forms a complex with the LDL receptor.

In a further embodiment of the invention, the assay is a Protein Misfolding Cyclic (PMCA) assay.

In a preferred embodiment of the invention, the Protein Misfolding Cyclic (PMCA) assay uses normal brain homogenate as a source of normal PrP^c and prion conversion factor.

In a preferred embodiment of the invention, the Protein Misfolding Cyclic (PMCA) assay uses cell lysates or lipid rafts extracted from prion infection sensitive neuroblasma

cells, such as line N2a, described in Example 2, and equivalent, as a source of normal PrP^c and prion conversion factor. Lipid raft fractions can also be purified directly from the brain to serve as a source of substrate for PMCA.

In another embodiment, the invention provides a use of a modulator, preferably an inhibitor or an antagonist, of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof for the preparation of a pharmaceutical preparation for the treatment of a prion disease, notably, bovine spongiform encephalopathy (BSE) and Creutzfeld-Jacob Disease (CJD). The modulator modifies for example the functions and/or properties of Apo B and/or Apo E or of a fragment thereof.

In a preferred embodiment of the invention, the modulator, preferably an inhibitor or an antagonist, of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof modifies, preferably inhibits the binding and/or the formation of a complex between Apolipoprotein B and/or Apolipoprotein E and the LDL receptor. An example of such modulator can be a LDL receptor modulator, a preferably LDL-receptor antagonist such as an anti-LDL receptor antibody.

In a preferred embodiment of the invention, the modulator is an antibody raised against Apolipoprotein B or against a fragment thereof.

In another preferred embodiment of the invention, the modulator is an antibody raised against Apolipoprotein B.

In another preferred embodiment of the invention, the modulator is an antibody raised against a fragment of Apolipoprotein B, which fragment is of, or about, a molecular weight selected from 30, 35 and 40 kDa.

In another preferred embodiment of the invention, the modulator is an antibody raised against a fragment of Apolipoprotein B, which fragment comprises a sequence selected

from fragments taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.

In a preferred embodiment of the invention, the peptide or protein is selected from Apolipoprotein B or a fragment thereof.

In another preferred embodiment of the invention, the peptide or protein is selected from Apolipoprotein E or a fragment thereof.

In a preferred embodiment of the invention, the peptide or protein contains the sequence of SEQ ID NO: 3.

In another preferred embodiment of the invention, the peptide or protein is a fragment which is of, or about, a molecular weight selected from 30, 35 and 40 kDa.

In another preferred embodiment of the invention, the peptide or protein is a fragment of Apolipoprotein B, comprising a sequence selected from fragments, taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.

In an embodiment of the invention, the invention provides a method for the diagnosis or detection of a prion disease within a subject suspected of suffering from such a disease which comprises (i) obtaining a sample from the subject; (ii) contacting a sample from said subject with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof (iii) contacting the sample obtained from step (ii) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample. The sample from the subject includes a biological extract from a mammal such as cell sample, genetic material, body fluid, brain homogenate, cells and lipid rafts.

In another embodiment of the invention, the invention provides a method of determining a marker that predisposes a subject to a prion disease, comprising (i)

obtaining a sample from the subject; (ii) measuring a level of a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment and mimetics thereof in said sample; (iii) contacting the sample obtained from step (ii) with PrP^{C} or PrP^{C} containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) correlating said level of protein obtained in said measuring step with the occurrence of a prion disease. The maker includes a biological parameter or value such as a genetic character, inherited protein mutation(s), blood level of a protein or an enzyme.

In another embodiment of the invention, the invention provides a method for the detection of PrP^{Sc} formation within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof; (iii) contacting the sample obtained from step (ii) with PrP^{C} or PrP^{C} containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample. The sample can be a biological preparation for which the presence of prion is to be detected for quality control reasons and/or a sample extracted from a subject that is suspected of suffering of such a disease, including a biological extract from a mammal such as cell sample, genetic material, body fluid, brain homogenate, cells and lipid rafts.

In another embodiment of the invention, the invention provides a method for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^{C} into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof (a) in the presence of said compound and (b) in the absence of said compound; (iii) contacting the sample obtained from step (i) a and (i) b, with PrP^{C} or PrP^{C} containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said compound. The modulator, includes antibodies, inhibitors of Apolipoproteins B and/or E binding, including binding to the LDL receptor, and/or secretion and/or synthesis.

Still another embodiment of the present invention, is a method for treating or preventing a prion disease such as bovine spongiform encephalopathy (BSE) and Creutzfeld-Jacob Disease (CJD), wherein the method comprises administering an effective dose of the above-mentioned modulator of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof, to a subject in the need thereof, wherein the subject can be human or animal.

In a preferred method of use of the modulators, preferably inhibitors, administration of the modulators is by injection or infusion, at periodic intervals. The administration of a compound of the invention may begin before any symptoms are detected in the patient, and should continue thereafter.

The above-mentioned modulatory compounds of the present invention may be administered by any means that achieves the intended purpose. For example, administration may be by a number of different routes including, but not limited to subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intra-cerebral, intrathecal, intranasal, oral, rectal, transdermal, intranasal or buccal. Preferably the compounds of the invention are administered by subcutaneous, intramuscular or intravenous injection or infusion.

Parenteral administration can be by bolus injection or by gradual perfusion over time. A typical regimen for preventing, suppressing, or treating prion related disorders, comprises either (1) administration of an effective amount in one or two doses of a high concentration of modulatory in the range of 0.5 to 10 mg of peptide, more preferably 0.5 to 10 mg of peptide, or (2) administration of an effective amount of the peptide in multiple doses of lower concentrations of modulatory compounds in the range of 10-1000 µg, more preferably 50-500 µg over a period of time up to and including several months to several years. It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The total dose required for each treatment may be administered by multiple doses or in a single dose.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients which are known in the art. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspension of the active compound as appropriate oily injections suspensions may be administered.

In another embodiment of the invention is provided an assay for the detection of the formation of PrP^{Sc} within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof; (iii) contacting the sample obtained from step (ii) with PrP^{C} or PrP^{C} containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample. The sample can be a biological preparation for which the presence of prion is to be detected for quality control reasons and/or a sample extracted from a subject that is suspected of suffering of such a disease, including a biological extract from a mammal such as cell sample, genetic material, body fluid, including blood, serum, plasma, brain homogenate, cells and lipid rafts.

In another embodiment of the invention, is provided a screening assay for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^{C} into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof (a) in the presence of said compound and (b) in the absence of said modulatory compound; (iii) contacting the sample obtained from step (i) a and (i) b with PrP^{C} or PrP^{C} containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said modulatory compound. The modulator, includes antibodies, inhibitors of Apolipoproteins B and/or E binding and/or secretion and/or synthesis.

In further embodiment of the invention, is provided a diagnostic kit for use in the assay of the invention, comprising a probe for receiving a sample and a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof. The kit of the invention comprises kits having multi-well microtitre plate and/or multi-well sonicator.

In a still further embodiment of the invention, is provided an apparatus for use in the methods of the invention or in the assays of the invention. The apparatus of the invention comprises apparatus that have a microtitre plate and/or multi-well sonicator.

In a preferred embodiment, the prion disease is bovine spongiform encephalopathy (BSE).

In a preferred embodiment, the prion disease is sporadic, variant, familial or iatrogenic Creutzfeld-Jacob Disease (CJD).

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions, which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

Brief description of the drawings:

Figure 1 shows *in vitro* prion replication on Hamster brain homogenate by PMCA assay in presence and absence of a cholesterol-depleting agent (Example 1§b). Samples contain 5% normal hamster brain homogenate incubated for 30 min at 4°C with 0, 5, 10 or 20 mM (final concentration) of methyl-β-cyclodextrin (MßCD). Aliquots of scrapie brain homogenate are added to reach a 3200- (top panel) and 12800-fold (bottom panel) dilution. Half of the samples are frozen immediately as a control

without amplification (PMCA "–") and the other half are subjected to 10 cycles of PMCA (PMCA "+"). Prion replication is evaluated by Western Blot after treatment of the samples with PK (100μg/ml for 60 min). The first lanes in each blot corresponds to the normal brain homogenate not treated with PK.

Figure 2 shows the effect of Apolipoproteins B, E and J *in vitro* prion replication on Hamster brain homogenate by PMCA assay (Example 1§c). Samples containing 5% normal hamster brain homogenate are incubated with different quantities of human ApoB (2A), human ApoE (2B) or murine ApoJ (2C) for 30 min at 4°C. Aliquots of scrapie brain homogenate are added to reach a 3200- (left panel) or 12800-fold (right panel) dilution. Half of the samples are frozen immediately as a control without amplification (PMCA "–") and the other half are subjected to 10 cycles of PMCA (PMCA "+"). Prion replication is evaluated by western blot after treatment of the samples with PK (100μg/ml for 60 min). The first lanes in each blot corresponds to the normal brain homogenate not treated with PK.

Figure 3 reports differential sensitivity of N2a sub-clones to infection by Scrapie revealed by exposure to anti-PrP 6H4 mabs (Example n° 2 §b). Proteinase K (PK) exposure shows were the PrP^{Sc} isoform (Proteinase K resistant) is present. The two sub-clones highlighted #23 and #60 are chosen respectively as representatives of prion infection resistant and sensitive cells. 'N2a' shows uninfected N2a cells processed in parallel. Controls for blotting and PK digestion show 1 μl normal or scrapie brain extract diluted in 80μl lysis buffer and processed in parallel.

Figure 4 shows the characterization of PrP in lipid rafts from sub-clones prion infection resistant (#23) and sensitive (#60) N2a (Example 2 §c). Figure 4A shows PrP quantification by Western blotting in lipid rafts which are extracted from prion infection resistant (#23) and sensitive (#60) cells. The distribution of PrP in the total extract (25μg loaded) (1), the sucrose sample layer after centrifigation (25μg loaded) (2) and the bouyant lipid raft fraction (4μg loaded) (3) are presented. **Figure 4B** shows PrP content and glycosylation pattern of the two sub-clones #23 and #60 by Western

blotting with anti-PrP. Three independent preparations of lipid rafts prion infection resistant (#23) and sensitive (#60) cells were analysed. Equal amounts (4μg) of rafts proteins were analysed in each case. **Figure 4C** shows the same membrane after stripping and re-probing with anti-actin which confirms the similarity in protein loading.

Figure 5 presents the *in vitro* conversion activity of lipid rafts from sub-clones prion infection resistant (#23) and sensitive (#60) N2a using PMCA (Example 2 §d). *Upper panel:* Lipid rafts are isolated from prion infection resistant (#23) and sensitive (#60) cells. Preparations are mixed in a ratio 100:1 with 10% RML brain homogenate and aliquots are frozen immediately, incubated for 15h at 37 °C or subjected to 15 cycles of PMCA. Lanes 1: initial mixture without PK digestion; lanes 2: initial mixture digested 10ug/ml PK 1hr 37 C; lanes 3: mixture incubated 37 °C PK digested as in lane 2; lanes 4: 15 cycles of PMCA followed by PK digestion as in lane 2. Lane 5 shows the migration and cross-reactivity with anti-PrP of PK alone. *Lower panel:* Following western blotting the membrane is stained with Coomasie blue to confirm that digestion with PK was complete.

Figure 6 shows the inhibitory effect on Prion replication in prion infection sensitive cells induced by Anti-hApoB polyclonal antibody (Example 2 §e).

Chronically infected #60 sensitive cells were cultured in 24 well culture dishes in the presence of increasing amounts (0-2mg/ml) of a goat polyclonal antibody against human ApoB (Chemicon) or against a corresponding series of naïve goat IgG. The level of PrP replication was determined by quantitative dot blotting and expressed as chemiluminescent intensity/mg protein. In the graph, for each antibody concentration the chemiluminescent intensity is expressed as a percentage of the value obtained without the antibody. Higher concentrations of anti-hApoB antibody have an inhibitory effect on PrP replication.

Figure 7 shows 2D separations of lipid raft proteins from N2a cells (Example 3). Lipid rafts are isolated from prion infection sensitive cells (#60) and 2 aliquots of 25 μg are precipitated with acetone and processed for 2D analysis min the 1st dimension spanning pH ranges 3-10 (7A) or 6-11 (7B). After SDS-PAGE separation in the second

dimension, gels are stained using the silver express kit (Invitrogen). Arrow indicates the same protein on both gels (7A and 7B). Proteins within the rectangle shown in B are compared between lipid raft from the prion infection sensitive sub-clone #60, (C) and resistant subclone #23, (D). Arrows indicate proteins which are more abundant in resistant cells.

Abbreviations:

Apo B (Apolipoprotein B; Apo E (apolipoprotein E); Apo J (Apolipoprotein J); BCA (Bicinchoninic Acid); CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate); CNS (central nervous system); BSE (bovine spongiform encephalopathy); CJD (Creutzfeldt-Jakob Disease); DIM (Detergent-Insoluble Membrane); DRM (Detergent-Resistant Membrane); DTT (1,4-Dithio-D,L-threitol); IPG (Immobilized PH Gradient); IEF (Isoelectric Focusing); FFI (Fatal Familial Insomnia); GSS (Gerstmann-Strassler-Scheinker Disease); hr (hour); HRP (Horseradish Peroxidase); kDa (KiloDalton); LDL (Low Density Lipoprotein); µg (microgram); µl (microliter); min (minute); MßCD (methyl-β-cyclodextrin); mM (millimolar); MS (mass spectrometry); PBS (Phosphate Buffered Sulfate); PK (proteinase K); PMCA (Protein Misfolding Cyclic Amplification); PMSF (Phenylmethanesulfonyl Fluoride); PrP (prion protein); PrP^{C} (normal, non-pathogenic conformer of PrP); PrP^{Sc} (pathogenic or "scrapie" isoform of PrP which is also the marker for prion diseases); PVDF (polyvinylidene difluoride); RPM (Rotation per minute); RML (Rocky Mountain Laboratory); RT-PCR (reverse transcriptase polymerase chain reaction); SDS (Sodium Dodecyl Sulfate); V (Volt); Vol. (volume).

EXAMPLES

The invention will be illustrated by means of the following examples which are not to be construed as limiting the scope of the invention.

The following examples illustrate preferred compounds and methods for determining their biological activities.

PrP scrapie used as infection innoculum is RML (Rocky Mountain Laboratory) strain.

Anti-PrP 6H4 monoclonal antibodies were purchased from Prionics.

Proteinase K was obtained from Boerhinger Ingelheim and Methyl.β.cyclodextrin from Sigma.

Purified and delipidated human Apolipoprotein B and Apolipoprotein E were obtained from Chemicon.

Human Anti-apo B antibodies were obtained from Chemicon.

Mouse neuroblasma N2a cell line was obtained from ATCC.

Murine Apo J was obtained in-house.

EXAMPLE 1: *In vitro* prion replication in brain homogenate through PMCA assay:

The influence of cholesterol and some of the apolipoproproteins on prion replication in vitro is analysed through a Protein Misfolding Cyclic Amplification assay (PMCA) (*Saborio et al., 2001*) where hamster brain homogenate is used as a source of PrP^C and conversion factors as follows.

a) Brain preparation:

Brains from healthy Syrian golden hamsters healthy or infected with the adapted scrapie strain 263 K are obtained after decapitation and immediately frozen in dry ice and kept at –80° until used. Brains are homogenized in PBS containing protease inhibitors (Complete™ cocktail from Boehringer Mannheim) at a 1x final concentration. Detergents (0.5% Triton X-100, 0.05% SDS, final concentrations) are added and samples clarified with low speed centrifugation (10000 g) for 1 min, using an Eppendorf centrifuge (model 5415).

Dilutions (3200-fold and 12800-fold) of the scrapie brain homogenate are added directly to the healthy brain homogenate to trigger prion replication. 60µl of these mixtures are frozen immediately and another 60µl are incubated at 37°C with agitation. Each hour a cycle of sonication (5 pulses of 1sec each) is done using a microsonicator (Bandelin Electronic, model Sonopuls) with the probe immersed in the sample and the power setting fixed at 40%. These cycles are repeated 10 times.

b) PMCA signal in presence and absence of a cholesterol-depleting agent:

Under these conditions a dramatic increase in the amount of PrP^{Sc} signal is observed after 10 cycles of PMCA (Figure 1, lanes 2 and 3). When normal brain

homogenate is treated during 30 min with 10 and 20mM (but not 5 mM) methyl-β–cyclodextrin (MßCD) a complete inhibition of prion replication is observed (Figure 1, lanes 6-9) as obtained in mouse models in cell cultures and in vitro, indicating that cholesterol depletion has a detrimental effect on prion replication *(Taraboulos et al., 1995).*

c) PMCA signal in presence of apolipoproteins

Purified delipidated human ApoB (Figure 2A) and human Apo E (Figure 2B) are respectively added to the PMCA preparation without cyclodextrin at different concentrations (8 and 16 µg for hApo B) and (1 and 10 µg for hApo E). Samples are incubated for 15 min at 4°C and thereafter half of each sample is frozen and the other half subjected to PMCA cycles.

An increase in prion replication in vitro is observed at both 3200-fold and 12800-fold dilutions of scrapie brain homogenate for both Apo B and Apo E. In contrast addition of Apolipoprotein J (at concentrations of 1, 2 and 4 µg), an Apolipoprotein component of HDL, has no effect on PMCA signal (Figure 2C).

These data show the effect of Apolipoproteins implicated B and E on prion conversion.

EXAMPLE 2: *In vitro* prion replication in lipid rafts from prion infection sensitive cells by PMCA:

The mouse neuroblastoma cell line N2a is used for their capability to be infected with PrP^{Sc}. *Baron et al., 2002* and *Enari et al., 2001* have shown that prion infection sensitive and prion infection resistant N2a sublines exist. Lipid rafts from the prion infection sensitive line are isolated and used as a substrate for PMCA assay. The effect of an apolipoprotein B antagonist on prion conversion is studied through the ability of apolipoprotein B antagonist to inhibit the prion replication ability of prion infection sensitive N2a cell lines.

a) N2a cell preparation:

Sub-clones of the parental mouse neuroblastoma N2a cell line are derived from single cells by limit dilution. A growing culture (Dulbecco's Modified Eagles Medium

(DMEM Gibco # 41966-029), containing 10% fetal calf serum (FCS) and supplemented with 2mM, L-glutamate and standard antibiotics (penicillin and streptomycin)) is diluted to a density of 5 cells/ml and 100µl is transferred to individual wells of a 96 well plate and cultured for 1 week.

The individual cultures are examined microscopically to determine those wells which contained a single focus of growing cells. The single cell derived cultures are then transferred to 24 well plates and serially passaged every 3-4 days at 1:15 dilution to maintain stocks. A total of 63 cultures are isolated and all tested for sensitivity to infection by the RML strain of PrP^{Sc}. To do this, 4µl of a 10% late stage infected brain extract is added per well of newly passaged cells, and the cultures are left for a further 4 days to reach 100 % confluence. Cells were serially passaged thereafter in the absence of PrP^{Sc}. Tests showed that all trace of the initial innoculum disappeared by passage 4.

At this and later passages individual cultures are tested for the presence of PrP^{Sc}.

b) Prion infection resistant cell isolation by Cell culture dot blotting:

The presence of PrP^{Sc} in the 63 individual cell cultures is tested by cell culture dot blotting procedure in which lysis and proteinase K (PK) digestion are carried out directly in the culture dish. PK resistant PrP^{Sc} is detected by dot blotting to PVDF membranes and exposure to anti-PrP antibody as follows:

Infected cells are grown for 3-4 days in 24-well plates and washed once with PBS. 40µl DNase1 (1000U/ml in H20) is added to each well at room temperature for 5min, followed by 40µl proteinase K solution (20µg/ml in 100mMTris/HCl pH 7.4, 300mM NaCl, 1% Triton-X100, 1% sodium deoxycholate). Plates are incubated at 37°C for 1hr with gentle agitation. Proteinase K digestion is stopped by addition of 2µl of a solution containing 80µg/ml PMSF, 10mmTris-HCl pH 8.0 and 1mg/ml bromophenol blue. 20µl aliquots are spotted onto PVDF membranes equilibrated with a degasses solution containing 192mM Glycine, 25mM Tris, 20% methanol. Membranes are then transferred to 3M guanidine Thiocyanate, 10mM Tris HCL pH 8.0 for 10min to denature proteins, rinsed in water and processed as for Western blotting using anti-PrP 6H4 (Prionics). Non-specific binding is blocked by incubation with 5% milk dissolved in PBS for 1hr. The membrane is then exposed to specific primary antibody anti-PrP 6H4, followed by HRP-conjugated secondary antibody each diluted as appropriate in

PBS, 0.3% Tween 20. Western blots are developed by ECL™ (Amersham) as directed according to the provider instructions.

The chemiluminescence signal from membranes is then analyzed directly using the Kodak Imagestation 440CF. The luminescence signal in each condition was normalized for possible differences in cell growth. Total protein content of a parallel lysate untreated with proteinase K is determined using the BCA assay (Biorad) and results are expressed as intensity/μg protein.

Of the 63 sub-clones analysed, 9 were found to be capable of replicating PrP^{Sc}, albeit with differing efficiencies (Figure 3). The remaining 54 sub-clones were resistant to infection. The most highly prion infection sensitive cell lines were selected for further study together with several prion infection resistant sub-clones with similar morphologies and doubling times. We have have selected two of these cell lines: #23, a prion infection resistant clone, and #60 a prion infection sensitive clone.

These two cell lines have been maintained in culture for over 1 year and have been infected with RML in many different occasions throughout this period: on every occasion sub-clone #60 was highly infectable whereas sub-clone #23 was totally resistant. Prion infection sensitive sub-clones could be maintained as a chronically infected cell culture by serial passaging at 1:15 or 1:20 dilution every 3 or 4 days respectively.

No evident morphological differences by microscopy were observed between the resistant or sensitive cells or between non-infected and infected cells.

To validate the clinical relevance of this cellular model of PrP relication, extracts of chronically infected N2a cells, or buffer alone, were injected into the hippocampus of normal mice by stereotactic injection. Injection of N2a extracts resulted in onset of clinical symptoms of scrapie after 140 days and premature death whereas mock injection had no effect on mouse physiology or life span. This indicates that the cell based model for prion replication using prion infection sensitive N2a cells generates infectious PrP scrapie, confirming that the conversion of PrP in cells is a good model for the process which occurs in vivo.

c) Lipid rafts isolation:

Procedures for isolating lipid rafts based on resistance to solubilization in cold Triton X-100 followed by flotation on sucrose gradients have been described by numerous laboratories *(Simons et al., 2000; Hooper et al., 1999)*. Lipid rafts from the two cell lines selected above are carried out as follows:

Subconfluent cultures of N2a cells are washed in PBS and collected by centrifugation 1000 x g for 5min. Typically 3 x 15cm dishes are pooled equivalent to approximately 8 $\times 10^7$ cells. The cell pellet is re-suspended in 1ml ice cold raft buffer (1% Triton in PBS, containing 10μM copper sulphate and a cocktail of complete protease inhibitors (Boehringer)). Cells are disrupted by seven passages through a 22G needle followed by incubation for 30 min at 4°C with gentle agitation. 2 volumes 60% sucrose in PBS is added and the lysate is transferred to a SW41 centrifuge tube. The lysate is carefully overlaid with 7 ml 35% sucrose and 1ml 15% sucrose both in PBS and centrifuged 20hr at 35,000 RPM. The lipid rafts are recovered in the top 1ml of the gradient. Membranes are concentrated by addition of 10 volumes cold PBS and centrifugation at 100,000g for 2hr. Alternatively for 2D gel electrophoresis, proteins from the lipid raft fraction are recovered by precipitation in the presence of 5 vol acetone for 2hr at –80°C. Acetone precipitates are collected by centrifugation 14000g 20min and washed twice in 70% ethanol.

In both sensitive and resistant cells around 1-2% of protein in the total lysate is recovered in the bouyant raft fraction. As shown by Western blotting (Figure 4A) while PrP is barely detectable in the total cell extract, it is highly enriched in rafts leaving the sample layer totally depleted of PrP following centrifugation.

Prion infection sensitive clone #60 and the prion infection resistant clone #23 are compared by westerm blotting with anti-PrP (Figure 4B). Three different independent pairs of raft preparations each containing 5μg total raft proteins are re-probed with anti-actin antibody which confirms the uniformity of PrP protein loading (Figure 4C).

The results indicate that the level of PrP in the lipid raft preparations from the two cell types is indistinguishable. Moreover the distribution between non-glycosylated mono- and di-glycosylated isoforms as well as the segregation to the detergent resistant

membrane fraction shown in Figure 4A is identical suggesting that none of these factors are likely to be responsible for the differing phenotypes.

PrP cDNA was amplified by RT-PCR from both cell lines as follows: Total RNA of N2a cells is prepared using Trizol (Gibco) and the mouse PrP cDNA is reversed transcribed with Omniscript (Qiagen) using the protocol supplied by the manufacturer. The specific primer for cDNA synthesis is 5' TCAATTGAAAGAGCTACAGGTG 3'. The prion cDNA is amplified using standard PCR conditions in the presence of primers 5' ACCAGTCCAATTTAGGAGAGCC 3' (top strand) and 5' AGACCACGAGAATGCGAAGG 3' (bottom strand). The PCR product was completely sequenced in the automated ABI3700 using the reagents and the protocol supplied by the manufacturer.

These data revealed that PrP mRNA is wild type in both cases and that both carry a Methionine at position 129, which in humans is the site for a frequent polymorphism.

Therefore, the expression levels, glycosylation patterns, intracellular localisation and primary sequences of PrP^C in both cell types is indistinguishable and thus that other cellular factors are responsible for the differential response to PrP^{Sc}.

d) *In vitro* cyclic amplification of protein misfolding (PMCA) in lipid rafts from prion infection sensitive cells:

Lipid rafts obtained at §c are isolated from prion infection sentive sub-clones, #60 sub-clones, collected by centrifugation as described above and resuspended in PMCA conversion buffer at a concentration of 2-2.5 mg/ml (PBS containing final concentration of 300mM NaCl, 0.5% Triton X100, 0.05% SDS).

A 10% extract of RML-infected mouse brain homogenate is added directly to the rafts preparation at a dilution of 1:100 based on protein content and aliquots of the mixture are either frozen immediately, incubated for 15hr at 37 °C or subjected to 15 cycles of PMCA (5 x 0.1 second pulses of sonication followed by incubation at 37 °C for 1hr).

Aliquots of 20μl sample are then treated with 10 μg /ml Proteinase K for 1hr 37 °C. Lipids are removed by precipitating PK-resistant proteins with 5 vol acetone for 2hr

at –80°C. Acetone precipitates are collected by centrifugation 14000g 20min, washed twice in 70% ethanol analysed by Western blotting with 6H4 anti-PrP (Figure 5).

Compared to the mixture without PK treatment (lanes 1 and 5) all digested samples show a shift in molecular weight characteristic of the N-terminally truncated PK resistant form $PrP_{27\text{-}30}$. It should be noted that the 6H4 antibody also has low level cross reactivity with PK which migrates at 30kDa, close to the di-glycosylated form of PK-digested PrP. Analysis of the data with this in mind shows that the initial level of PK-resistant PrP derived from the diluted brain extract, which is present in the non-amplified mixtures, is barely detectable under these conditions (lanes 2).

A slight increase in signal is seen when the prion infection sensitive (#60) DRM is incubated at 37 °C for 15 hr (lane 3 from #60), however the most dramatic increase in PK-resistant PrP is seen when this sample is subjected to 15 cycles of PMCA (lane 4 from #60). This indicates that all factors required for conversion of PrP^{C} to PrP^{Sc} are resident in the lipid rafts from the prion infection sensitive N2a cells. Interestingly, in the parallel analysis in which the DRM from the prion infection resistant cell line #23 was used, no amplification in vitro was observed (lane 4 from #23) indicating that the capacity of the lipid rafts to convert the prion protein in vitro reflects the activity observed in the intact cells.

e) Effect of antibody raised against apolipoprotein B on prion replication by prion infection sensitive N2a cells:

Chronically infected sensitive cells were cultured in 24-well dishes in the presence of a goat polyclonal antibody raised against human Apo B (Chemicon) at increasing concentrations from 0 to 2mg/ml in DMEM Gibco # 41966-029, containing 1 x B27 supplements (Gibco #17504-044) and standard antibiotics (penicillin and streptomycin)

A parallel series of cultures was incubated in the presence of the same concentration range of total IgG from a naïve goat. The results show that concentrations of anti-hApoB antibody above 0.5mg/ml result in progressive inhibition of PrP replication as revealed by quantitative dot blotting (Figure 6).

EXAMPLE 3: Proteomics analysis of lipid rafts of prion infection resistant and sensitive cells:

Since the two cell preparations are indistinguishable in terms of their PrP content a more complete protein comparison using 2D gel electrophoresis was performed to show differences in other proteins that might underline the difference in conversion activity between the two sub-clones.

2D gel preparations are prepared as follows:

Acetone precipitated proteins (see §c) are re-suspended in 20µl 1% SDS, 0.23% DTT and heated to 95°C for 5min. After the preparation is cooled to room temperature, 25µl of a solution (9M urea, 4% CHAPS, 65mM DTT, 35mM Tris base) is added.

Fifteen minutes later, 85µl of a solution containing 7M urea, 2M thiourea, 4% CHAPS, 100mM DTT is further added to the mixture. After a further 15 min, non-solubilized material is removed by centrifugation at 14000 RPM during 5min and the supernatant is applied directly to a 7cm IPG strip and left to re-hydrate overnight. For IEF the voltage is progressively increased from 300V to 3.5kV and electrophoresed for a total of 20kVh. Proteins are resolved in the second dimension using single well 4-12% gradient gels (Novex) and stained using the silver express kit (Invitogen) according to the instructions supplied.

Analysis by 2D gels reveals the fraction of protein that is recovered in the lipid rafts (approximately 1-2% protein in the N2a cell lysate) as a reproducible subset of total cell proteins in which several hundred species can be visualized following silver staining (Figure 7A and B).

The 2D patterns are compared between preparations isolated from the prion infection sensitive and resistant cells. The analysis is focused on several proteins identified in the basic range of the gel, which are more abundant in DRMs from prion infection resistant cells (arrows in Figure 7C and D).

Following preparative scale electrophoresis, the two proteins indicated by arrows are excised and processed for MS sequencing. From both proteins an identical tryptic peptide is found with a monoisotopic mass of 1234.6. The N-terminal sequence of this tryptic peptide is: ENFAGEATLQR (SEQ ID NO: 3). All amino acids in the peptide are identified in the MS/MS spectrum of doubly charged precursor ion at m/z 618.30 And through its Mascot analysis.

Database searching identified this protein unambiguously as Apolipoprotein B (Apo B). Since the molecular weight of full length Apo B is in excess of 500 kDa while these two spots migrate with estimated molecular weights of 40kDa and 30kDa, we presume that the latter are fragments generated either in the cell or during sample preparation. The sequence corresponds to amino acids 3548-3558 of the human Apo B protein, which is present only in ApoB-100 and not in the truncated ApoB-48 form.

These data suggest that fragments of a molecular weight of or about 30 to 40 kDa comprising the sequence of SEQ ID NO: 3 may have a role in the prion conversion pathway.

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Claims

1. Use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof in an assay for the detection of the formation of PrP^{sc} in a sample.

2. Use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof in a screening assay for identifying compounds that modulate the conversion of PrP^{c} into PrP^{sc}.

3. Use according to claims 1 or 2 wherein the peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof forms a complex with the LDL receptor.

4. Use according to any of the preceding claims wherein the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay.

5. Use according to any of the preceding claims wherein the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay using normal brain homogenate as a source of normal PrP^{C} and substrate.

6. Use according to claims 1 to 4 wherein the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay using lipid rafts from infection sensitive neuroblasma cell line N2a as a source of normal PrP^{C} and substrate.

7. Use of a modulator of a protein or a peptide selected from Apolipoprotein B, and fragment thereof, Apolipoprotein E and a fragment thereof for the preparation of a pharmaceutical preparation for the treatment of a prion disease.

8. Use according to claim 7 wherein the modulator antagonizes the formation of a complex between Apolipoprotein B and/or Apolipoprotein E with the LDL receptor.

9. Use according to claims 7 or 8 wherein the modulator is an antibody raised against Apolipoprotein B or a fragment thereof.

10. Use according to claims 7 or 8 wherein the modulator is an antibody raised against Apolipoprotein E or a fragment thereof.

11. Use according to any of the preceding claims wherein the peptide or the protein is Apolipoprotein B or a fragment thereof.

12. Use according to any of the preceding claims wherein the peptide of the protein contains the sequence of SEQ ID NO: 3.

13. Use according to any of the preceding claims wherein the peptide or the protein is of a molecular weight selected from 30 and 40 kDa and which sequence is selected from fragments of Apolipoprotein B taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.

14. Use according to to any of the preceding claims wherein the peptide or the protein is Apolipoprotein E or a fragment thereof.

15. Use according to any one of claims 7 to 14 wherein the prion disease is bovine spongiform encephalopathy (BSE).

16. Use according to any one of claims 7 to 14 wherein the prion disease is a Creutzfeld-Jacob Disease (CJD).

17. A method for the diagnosis or detection of a prion disease within a subject suspected of suffering from such a disease which comprises (i) obtaining a sample from the subject; (ii) contacting a sample from said subject with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof; (iii) contacting the mixture obtained in

step (ii) with PrP^C or PrP^C containing mixtures; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample.

18. A method of determining a marker that predisposes a subject to a prion disease, comprising (i) obtaining a sample from the subject; (ii) measuring a level of a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof in said sample; and (iii) correlating said level of protein obtained in said measuring step with the occurrence of a prion disease.

19. A method for the detection of PrP^{Sc} within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof; (ii) contacting sample obtained in (i) with PrP^C or PrP^C containing mixtures; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

20. A method according to any one of claims 17 to 19 wherein the prion disease is bovine spongiform encephalopathy (BSE).

21. A method according to any one of claims 17 to 19 wherein the prion disease is a Creutzfeld-Jacob disease.

22. A method for identifying a compound which modulates the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof (a) in the presence of said modulatory compound and (b) in the absence of said compound; (ii) contacting the mixtures obtained in step (i) a and (i) b with PrP^C or PrP^C containing mixtures; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said modulatory compound and (b) in the absence of said modulatory compound.

23. An assay for the detection of PrP^{Sc} in a sample within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from

Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof; (ii) contacting the mixture obtained in step (i) with PrP^C or PrP^C containing mixtures; (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

24. A screening assay for identifying a compound which modulates the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof (a) in the presence of said modulatory compound and (b) in the absence of said modulatory compound; (ii) contacting the mixtures obtained in step (i) a and (i) b with PrP^C or PrP^C containing mixtures; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said modulatory compound.

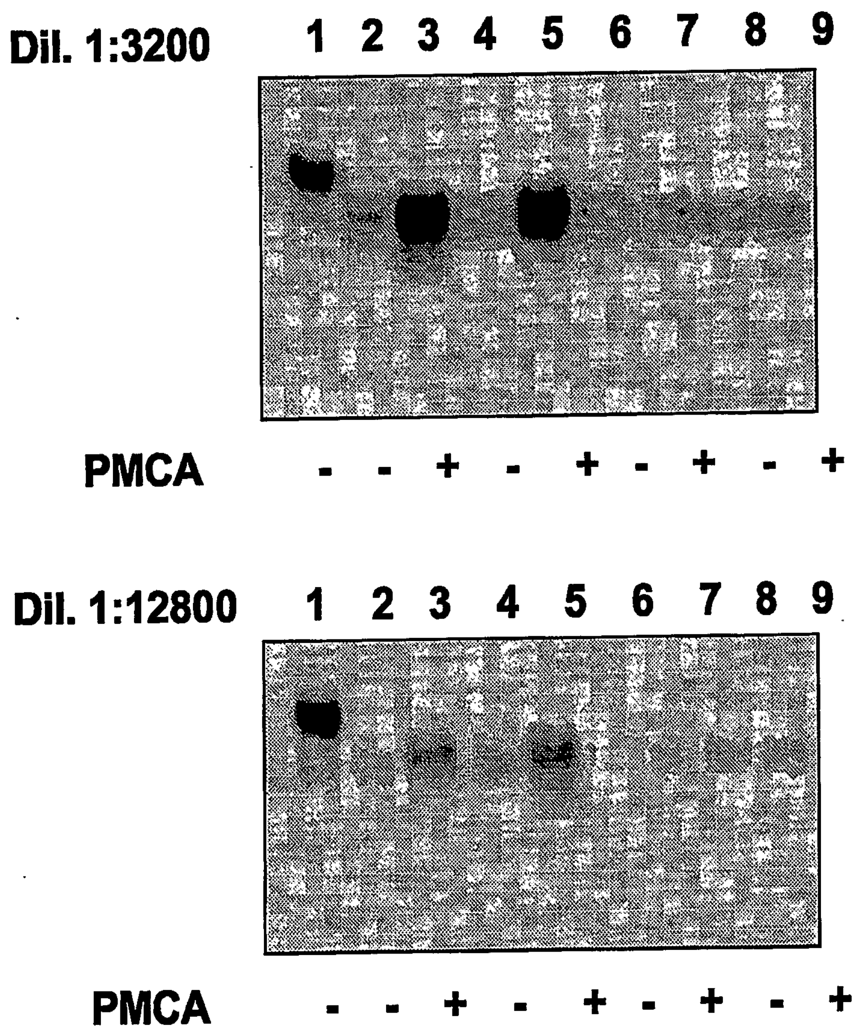
25. A diagnostic kit for use in the assay according to claim 23, comprising a probe for receiving a sample and a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof.

26. An apparatus for use in the method of any one of claims 17 to 22 or the assay of any one claims 23 to 24.

Abstract

The use of apolipoprotein B or apolipoprotein E or fragments thereof is provided for diagnostic, detection, prognostic and therapeutic applications in prion diseases. More specifically, the invention provides the use of apolipoprotein B or apolipoprotein E or fragments thereof for modulating or identifying modulators of the prion protein replication which are implicated in the pathogenesis of transmissible spongiform encephalopathies and other prion diseases.

Figure 1



Lanes	MßCD concentration, mM
2, 3	0
4, 5	5
6, 7	10
8,9	20

Figure 2A

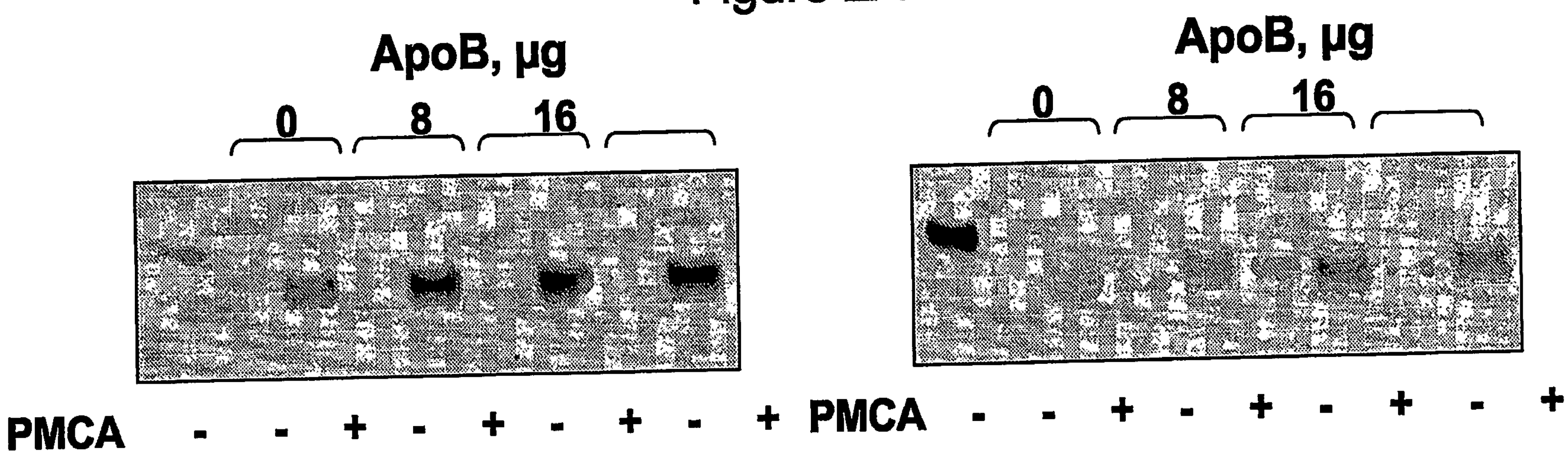


Figure 2B

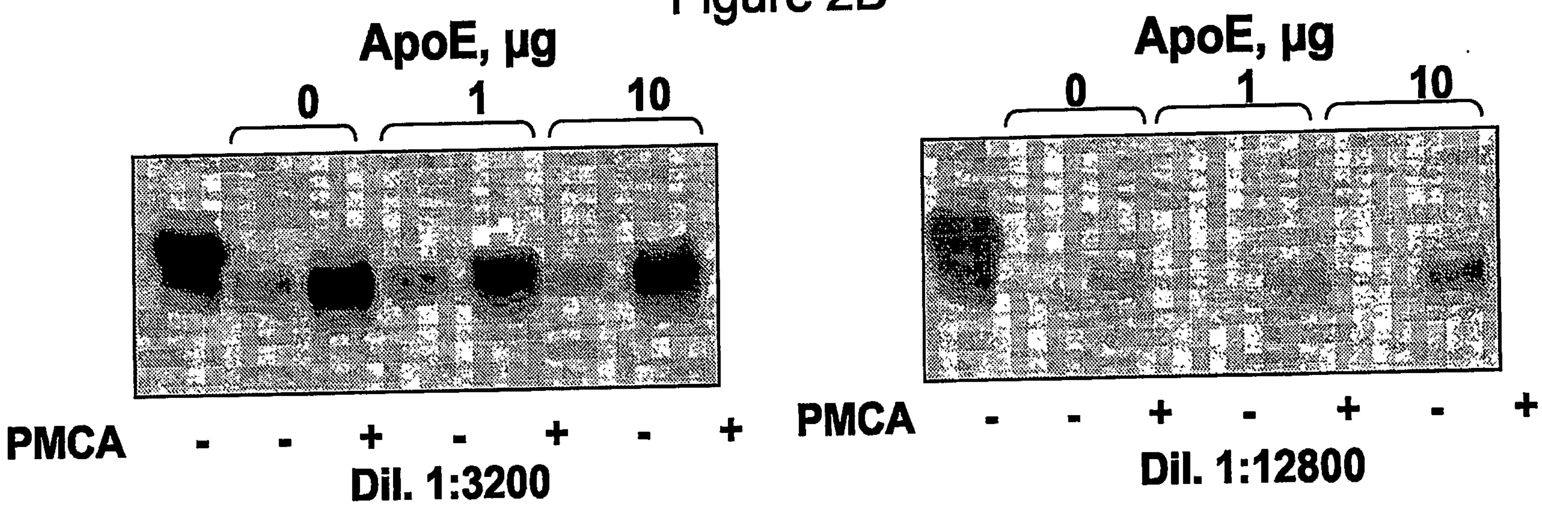


Figure 2C

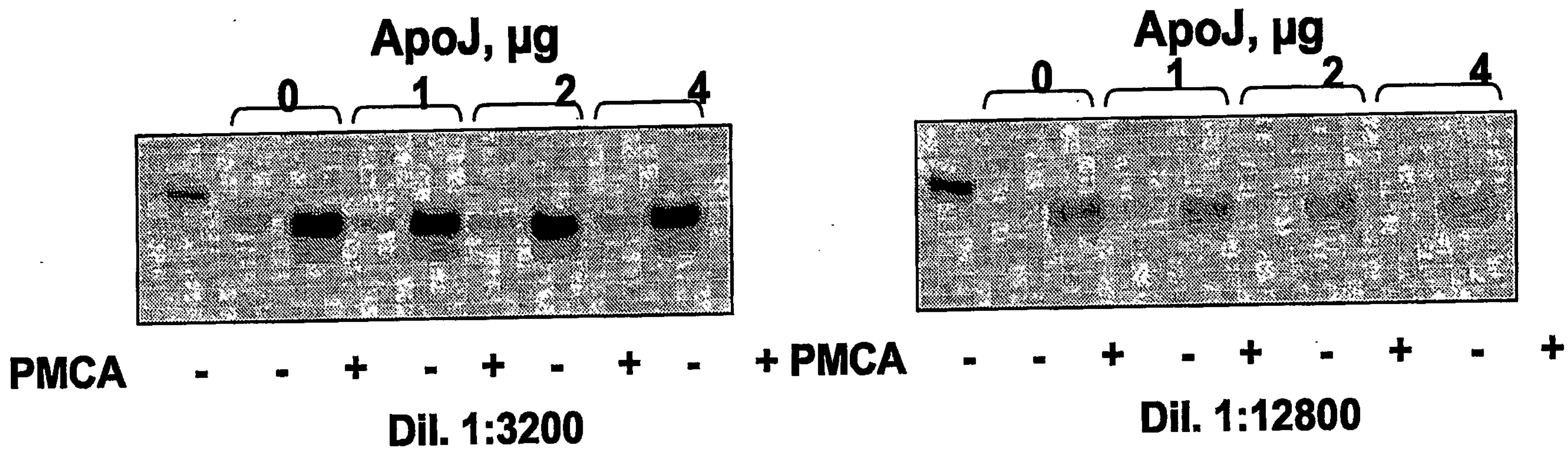
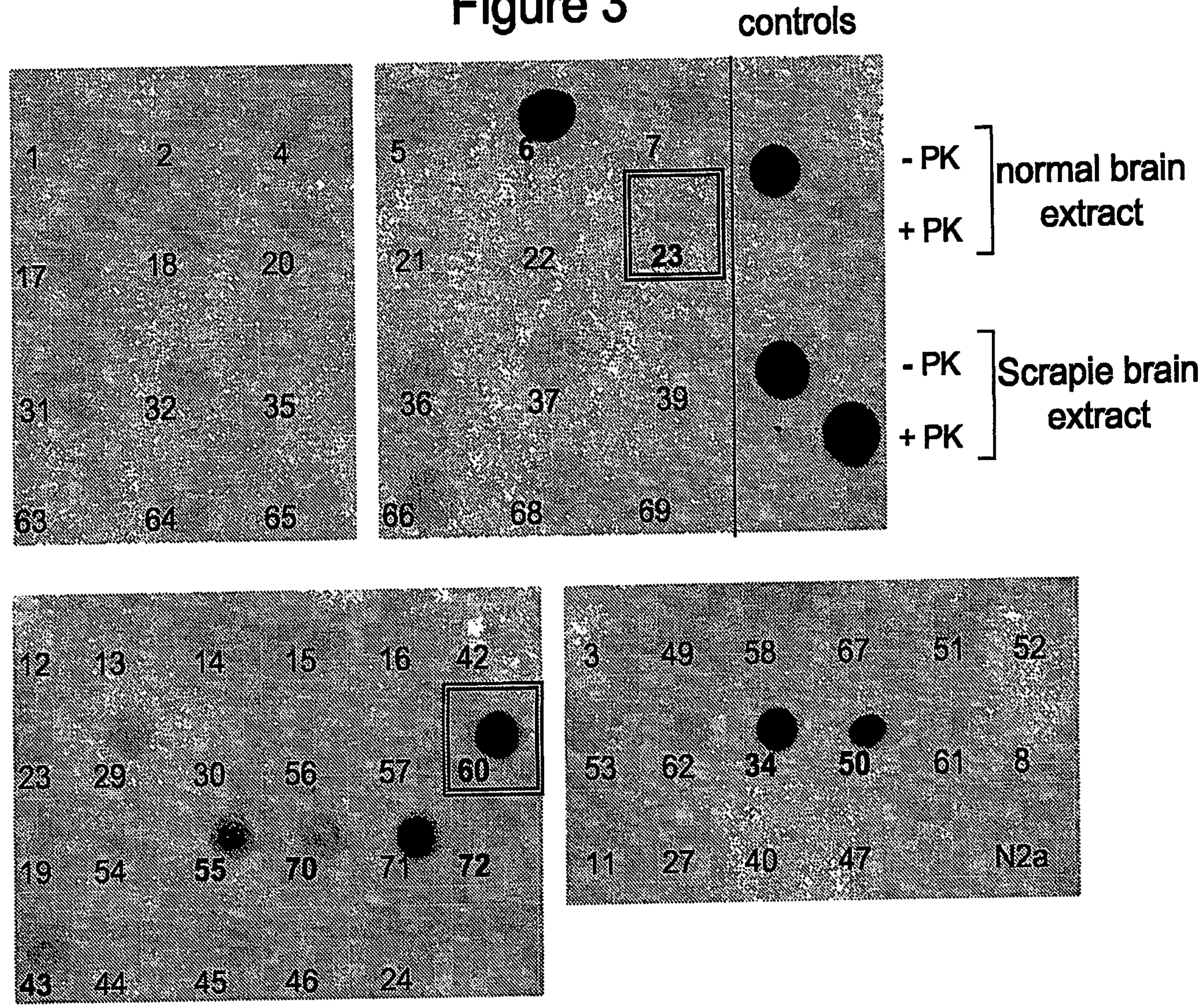


Figure 3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2
4
17
18
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31
32
35
63
64
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22
23
36
37
39
66
68
69
- PK
+ PK
normal brain extract
- PK
+ PK
Scrapie brain extract
12
13
14
15
16
42
23
29
30
56
57
60
19
54
55
70
71
72
43
44
45
46
24
3
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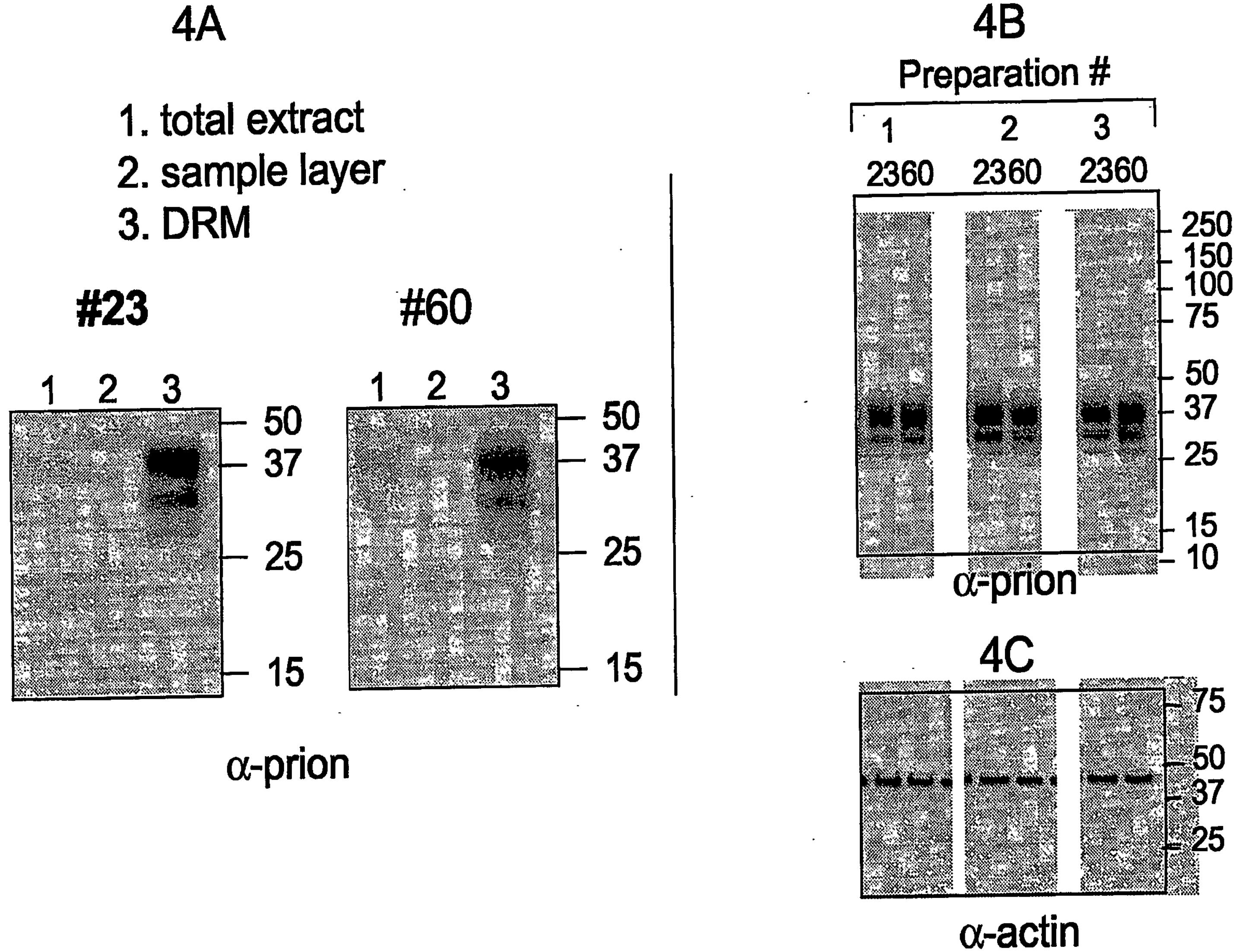
52
53
62
34
50
61
8
11
27
40
47
N2a

Figure 4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1. total extract
2. sample layer
3. DRM
#23
#60
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2
3
50
37
25
15
α-prion
4B
Preparation #
1
2
3
2360
2360
2360
250
150
100
75
50
37
25
15
10
α-prion
4C
75
50
37
25
α-actin

Figure 5

1. frozen - w/o PK
2. frozen - PK digested
3. 37°C 15h - PK digested
4. PMCA 15 cycles - PK digested

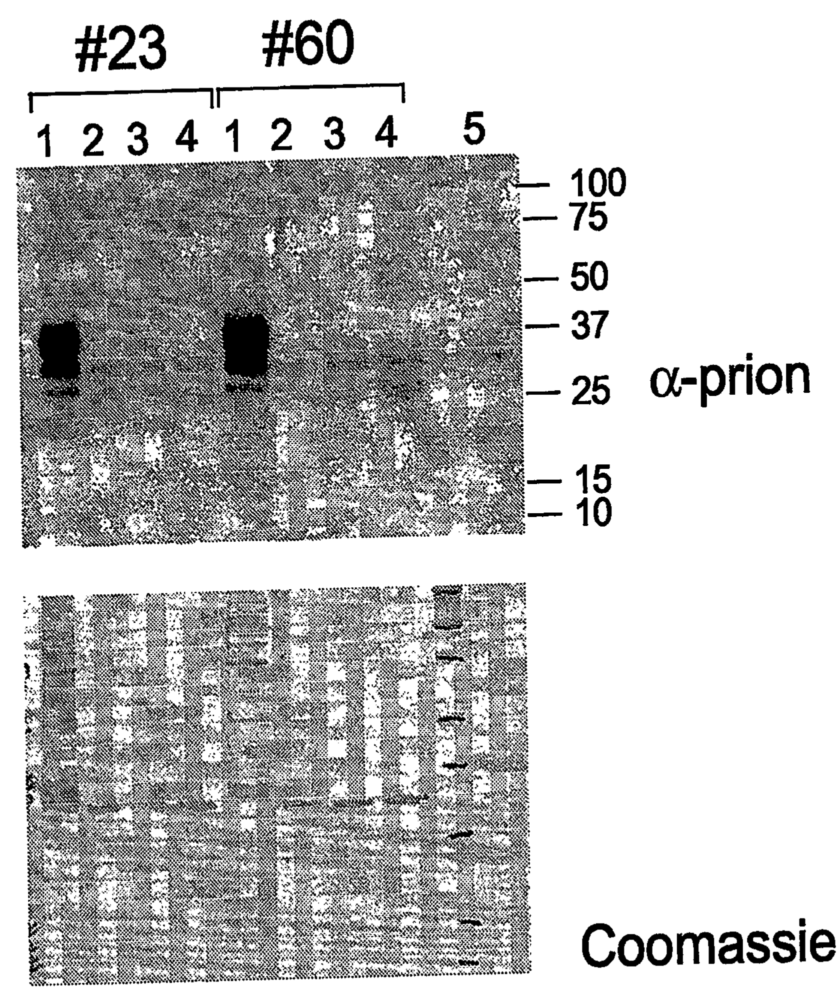
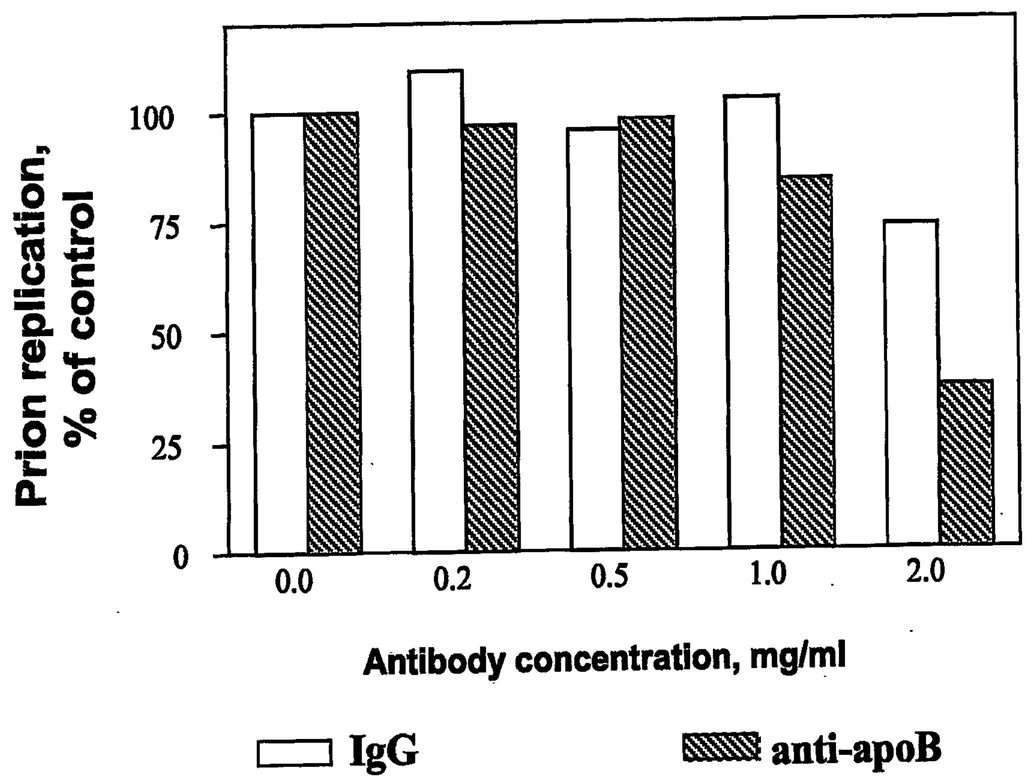
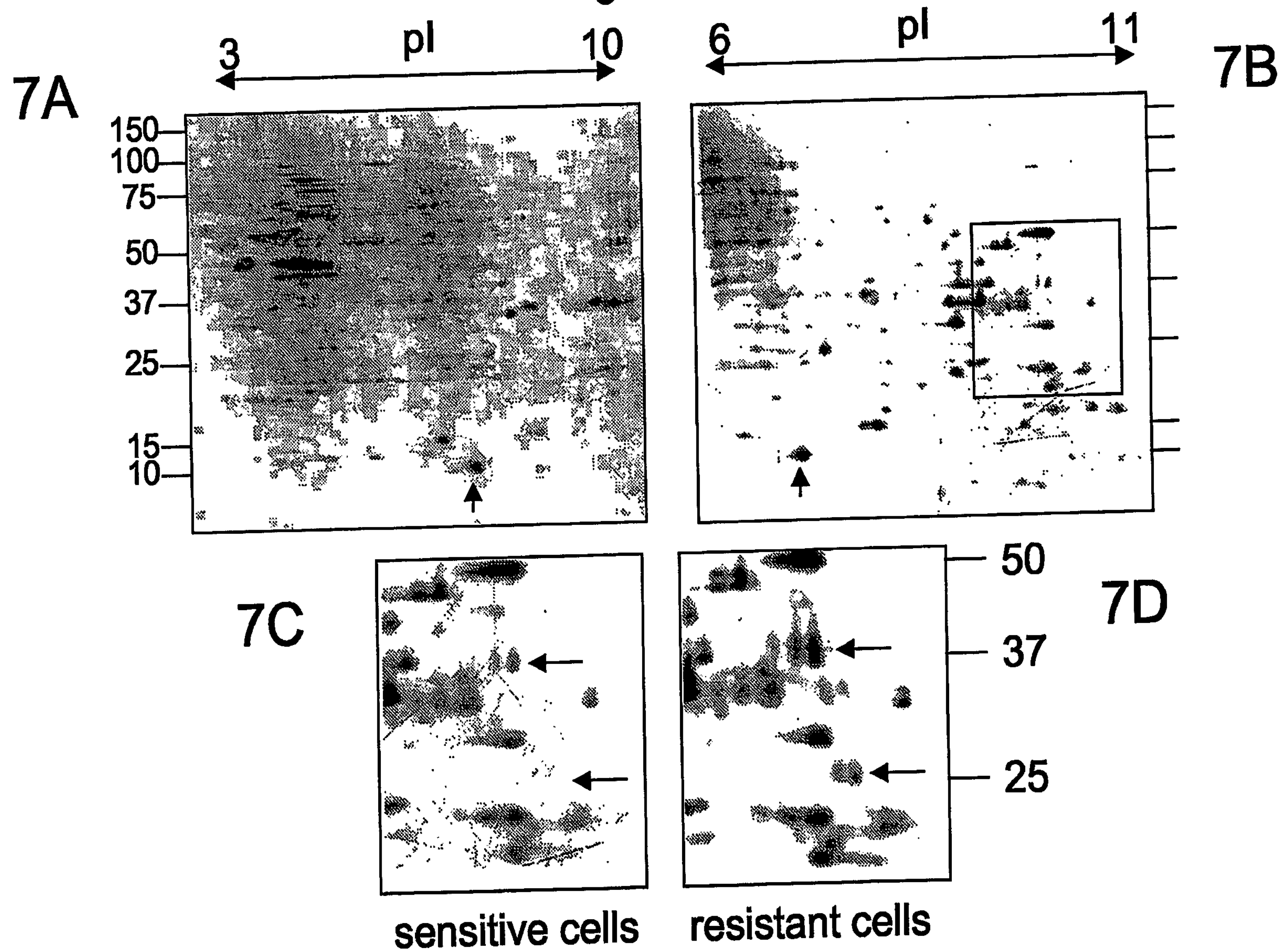


Figure 6



Prion replication, % of control
100
75
50
25
0
0.0
0.2
0.5
1.0
2.0
Antibody concentration, mg/ml
IgG
anti-apoB

Figure 7


7A
3
pI
10
150
100
75
50
37
25
15
10
6
pI
11
7B
7C
7D
50
37
25
sensitive cells
resistant cells

SEQUENCE LISTING

<110> APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.

<120> USE OF PRION CONVERSION MODULATING AGENTS

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<160> 3

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        35                  40                  45

Leu Arg Lys Tyr Thr Tyr Asn Tyr Glu Ala Glu Ser Ser Ser Gly Val
    50                  55                  60

Pro Gly Thr Ala Asp Ser Arg Ser Ala Thr Arg Ile Asn Cys Lys Val
65                  70                  75                  80

Glu Leu Glu Val Pro Gln Leu Cys Ser Phe Ile Leu Lys Thr Ser Gln
                85                  90                  95

Cys Thr Leu Lys Glu Val Tyr Gly Phe Asn Pro Glu Gly Lys Ala Leu
            100                 105                 110

Leu Lys Lys Thr Lys Asn Ser Glu Glu Phe Ala Ala Ala Met Ser Arg
        115                 120                 125

Tyr Glu Leu Lys Leu Ala Ile Pro Glu Gly Lys Gln Val Phe Leu Tyr
    130                 135                 140

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            180                     185                     190

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        195                     200                     205

Leu Gly Gln Cys Asp Arg Phe Lys Pro Ile Arg Thr Gly Ile Ser Pro
    210                     215                     220

Leu Ala Leu Ile Lys Gly Met Thr Arg Pro Leu Ser Thr Leu Ile Ser
225                     230                     235                     240

Ser Ser Gln Ser Cys Gln Tyr Thr Leu Asp Ala Lys Arg Lys Hi s Val
                245                     250                     255

Ala Glu Ala Ile Cys Lys Glu Gln His Leu Phe Leu Pro Phe Ser Tyr
            260                     265                     270

Asn Asn Lys Tyr Gly Met Val Ala Gln Val Thr Gln Th r Leu Lys Leu
        275                     280                     285

Glu Asp Thr Pro Lys Ile Asn Ser Arg Phe Phe Gly Glu Gly Thr Lys
    290                     295                     300

Lys Met Gly Leu Ala Phe Glu Ser Thr Lys Se r Thr Ser Pro Pro Lys
305                     310                     315                     320

Gln Ala Glu Ala Val Leu Lys Thr Leu Gln Glu Leu Lys Lys Leu Thr
                325                     330                     335

Ile Ser Glu Gln Asn Ile Gln Arg Al a Asn Leu Phe Asn Lys Leu Val
            340                     345                     350

Thr Glu Leu Arg Gly Leu Ser Asp Glu Ala Val Thr Ser Leu Leu Pro
        355                     360                     365

Gln Leu Ile Glu Val Ser Se r Pro Ile Thr Leu Gln Ala Leu Val Gln
    370                     375                     380

Cys Gly Gln Pro Gln Cys Ser Thr His Ile Leu Gln Trp Leu Lys Arg
385                     390                     395                     400

Val His Ala Asn Pro o Leu Leu Ile Asp Val Val Thr Tyr Leu Val Ala
                405                     410                     415
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Asp Glu Asp Tyr Thr Tyr Leu Ile Leu Arg Val Ile Gly Asn Met Gly
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Cys Val Gln Ser Thr Lys Pro Ser Leu Met Ile Gln Lys Ala Ala Ile
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Gln Ala Leu Arg Lys Met Glu Pro Lys Asp Lys Asp Gln Glu Val Leu
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645 650 655

Gly Asn Leu Ile Phe Asp Pro Asn Asn Tyr Leu Pro Lys Glu Ser Met
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Leu Lys Thr Thr Leu Thr Ala Phe Gly Phe Ala Ser Ala Asp Leu Ile
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Glu Ile Gly Leu Glu Gly Lys Gly Phe Glu Pro Thr Leu Glu Ala Leu
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Trp Val Asn Gly Gln Val Pro Asp Gly Val Ser Lys Val Leu Val Asp
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740 745 750

Gly Ile Met Leu Ser Val Glu Lys Leu Ile Lys Asp Leu Lys Ser Lys
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1970 1975 1980

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1985 1990 1995

Tyr Asn Thr Lys Asp Lys Ile Gly Val Glu Leu Thr Gly Arg Thr
2000 2005 2010

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2015 2020 20 25

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2060 2065 2070

Phe Glu Thr Leu Gln Glu Tyr Phe Glu Arg Asn Arg Gln Thr Ile

2075 2080 2085

Ile Val Val Val Glu Asn Val Gln Arg Asn Leu Lys His Ile Asn

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Pro Gln Gln Ala Asn Asp Tyr Leu Asn Ser Phe Asn Trp Glu Arg

2120 2125 2130

Gln Val Ser His Ala Lys Glu Lys Leu Thr Ala Leu Thr Lys Lys

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Tyr Arg Ile Thr Glu Asn Asp Ile Gln Ile Ala Leu Asp Asp Ala

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Lys Thr Ile His Asp Leu His Leu Phe Ile Glu Asn Ile Asp Phe

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Trp Thr Leu Ala Ala Lys Asn Leu Thr Asp Phe Ala Glu Gln Tyr
2555 2560 2565

Ser Ile Gln Asp Trp Ala Lys Arg Met Lys Ala Leu Val Glu Gln
2570 2575 2580

Gly Phe Thr Val Pro Glu Ile Lys Thr Ile Leu Gly Thr Met Pro
2585 2590 2595

Ala Phe Glu Val Ser Leu Gln Ala Leu Gln Lys Ala Thr Phe Gln
2600 2605 2610

Thr Pro Asp Phe Ile Val Pro Leu Thr Asp Leu Arg Ile Pro Ser
2615 2620 2625

Val Gln Ile Asn Phe Lys Asp Leu Lys Asn Ile Lys Ile Pro Ser
2630 2635 2640

Arg Phe Ser Thr Pro Glu Phe Thr Ile Leu Asn Thr Phe His Ile
2645 2650 2655

Pro Ser Phe Thr Ile Asp Phe Val Glu Met Lys Val Lys Ile Ile
2660 2665 2670

Arg Thr Ile Asp Gln Met Gln Asn Ser Glu Leu Gln Trp Pro Val
2675 2680 2685

Pro Asp Ile Tyr Leu Arg Asp Leu Lys Val Glu Asp Ile Pro Leu
2690 2695 2700

Ala Arg Ile Thr Leu Pro Asp Phe Arg Leu Pro Glu Ile Ala Ile
2705 2710 2715

Pro Glu Phe Ile Ile Pro Thr Leu Asn Leu Asn Asp Phe Gln Val
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Pro Asp Leu His Ile Pro Glu Phe Gln Leu Pro His Ile Ser His
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Thr Ile Glu Val Pro Thr Phe Gly Lys Leu Tyr Ser Ile Leu Lys
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Ile Gln Ser Pro Leu Phe Thr Leu Asp Ala Asn Ala Asp Ile Gly
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Asn Gly Thr Thr Ser Ala Asn Glu Ala Gly Ile Ala Ala Ser Ile
2780 2785 2790

Thr Ala Lys Gly Glu Ser Lys Leu Glu Val Leu Asn Phe Asp Phe
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Gln Ala Asn Ala Gln Leu Ser Asn Pro Lys Ile Asn Pro Leu Ala
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Leu Lys Glu Ser Val Lys Phe Ser Ser Lys Tyr Leu Arg Thr Glu
2825 2830 2835

His Gly Ser Glu Met Leu Phe Phe Gly Asn Ala Ile Glu Gly Lys
2840 2845 2850

Ser Asn Thr Val Ala Ser Leu His Thr Glu Lys Asn Thr Leu Glu
2855 2860 2865

Leu Ser Asn Gly Val Ile Val Lys Ile Asn Asn Gln Leu Thr Leu
2870 2875 2880

Asp Ser Asn Thr Lys Tyr Phe His Lys Leu Asn Ile Pro Lys Leu
2885 2890 2895

Asp Phe Ser Ser Gln Ala Asp Leu Arg Asn Glu Ile Lys Thr Leu
2900 2905 2910

Leu Lys Ala Gly His Ile Ala Trp Thr Ser Ser Gly Lys Gly Ser
2915 2920 2925

Trp Lys Trp Ala Cys Pro Arg Phe Ser Asp Glu Gly Thr His Glu
2930 2935 2940

Ser Gln Ile Ser Phe Thr Ile Glu Gly Pro Leu Thr Ser Phe Gly
2945 2950 2955

Leu Ser Asn Lys Ile Asn Ser Lys His Leu Arg Val Asn Gln Asn

2960 2965 2970

Leu Val Tyr Glu Ser Gly Ser Leu Asn Phe Ser Lys Leu Glu Ile
2975 2980 2985

Gln Ser Gln Val Asp Ser Gln His Val Gly His Ser Val Leu Thr
2990 2995 3000

Ala Lys Gly Met Ala Leu Phe Gly Glu Gly Lys Ala Glu Phe Thr
3005 3010 3015

Gly Arg His Asp Ala His Leu Asn Gly Lys Val Ile Gly Thr Leu
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Lys Asn Ser Leu Phe Phe Ser Ala Gln Pro Phe Glu Ile Thr Ala
3035 3040 3045

Ser Thr Asn Asn Glu Gly Asn Leu Lys Val Arg Phe Pro Leu Arg
3050 3055 3060

Leu Thr Gly Lys Ile Asp Phe Leu Asn Asn Tyr Ala Leu Phe Leu
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Ser Pro Ser Ala Gln Gln Ala Ser Trp Gln Val Ser Ala Arg Phe
3080 3085 3090

Asn Gln Tyr Lys Tyr Asn Gln Asn Phe Ser Ala Gly Asn Asn Glu
3095 3100 3105

Asn Ile Met Glu Ala His Val Gly Ile Asn Gly Glu Ala Asn Leu
3110 3115 3120

Asp Phe Leu Asn Ile Pro Leu Thr Ile Pro Glu Met Arg Leu Pro
3125 3130 3135

Tyr Thr Ile Ile Thr Thr Pro Pro Leu Lys Asp Phe Ser Leu Trp
3140 3145 3150

Glu Lys Thr Gly Leu Lys Glu Phe Leu Lys Thr Thr Lys Gln Ser
3155 3160 3165

Phe Asp Leu Ser Val Lys Ala Gln Tyr Lys Lys Asn Lys His Arg
3170 3175 3180

His Ser Ile Thr Asn Pro Leu Ala Val Leu Cys Glu Phe Ile Ser
3185 3190 3195

Gln Ser Ile Lys Ser Phe Asp Arg His Phe Glu Lys Asn Arg Asn
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Asn Ala Leu Asp Phe Val Thr Lys Ser Tyr Asn Glu Thr Lys Ile
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Lys Phe Asp Lys Tyr Lys Ala Glu Lys Ser His Asp Glu Leu Pro
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Arg Thr Phe Gln Ile Pro Gly Tyr Thr Val Pro Val Val Asn Val
3245 3250 3255

Glu Val Ser Pro Phe Thr Ile Glu Met Ser Ala Phe Gly Tyr Val
3260 3265 3270

Phe Pro Lys Ala Val Ser Met Pro Ser Phe Ser Ile Leu Gly Ser
3275 3280 3285

Asp Val Arg Val Pro Ser Tyr Thr Leu Ile Leu Pro Ser Leu Glu
3290 3295 3300

Leu Pro Val Leu His Val Pro Arg Asn Leu Lys Leu Ser Leu Pro
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His Phe Lys Glu Leu Cys Thr Ile Ser His Ile Phe Ile Pro Ala
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Met Gly Asn Ile Thr Tyr Asp Phe Ser Phe Lys Ser Ser Val Ile
3335 3340 3345

Thr Leu Asn Thr Asn Ala Glu Leu Phe Asn Gln Ser Asp Ile Val
3350 3355 3360

Ala His Leu Leu Ser Ser Ser Ser Ser Val Ile Asp Ala Leu Gln
3365 3370 3375

Tyr Lys Leu Glu Gly Thr Thr Arg Leu Thr Arg Lys Arg Gly Leu
3380 3385 3390

Lys Leu Ala Thr Ala Leu Ser Leu Ser Asn Lys Phe Val Glu Gly
3395 3400 3405

Ser His Asn Ser Thr Val Ser Leu Thr Thr Lys Asn Met Glu Val
3410 3415 3420

Ser Val Ala Lys Thr Thr Lys Ala Glu Ile Pro Ile Leu Arg Met
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Asn Phe Lys Gln Glu Leu Asn Gly Asn Thr Lys Ser Lys Pro Thr
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Val Ser Ser Ser Met Glu Phe Lys Tyr Asp Phe Asn Ser Ser Met
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Leu Tyr Ser Thr Ala Lys Gly Ala Val Asp His Lys Leu Ser Leu
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Glu Ser Leu Thr Ser Tyr Phe Ser Ile Glu Ser Ser Thr Lys Gly
3485 3490 3495

Asp Val Lys Gly Ser Val Leu Ser Arg Glu Tyr Ser Gly Thr Ile
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Ala Ser Glu Ala Asn Thr Tyr Leu Asn Ser Lys Ser Thr Arg Ser
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Ser Val Lys Leu Gln Gly Thr Ser Lys Ile Asp Asp Ile Trp Asn
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Leu Glu Val Lys Glu Asn Phe Ala Gly Glu Ala Thr Leu Gln Arg
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Ile Tyr Ser Leu Trp Glu His Ser Thr Lys Asn His Leu Gln Leu
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Glu Gly Leu Phe Phe Thr Asn Gly Glu His Thr Ser Lys Ala Thr
3575 3580 3585

Leu Glu Leu Ser Pro Trp Gln Met Ser Ala Leu Val Gln Val His
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Ala Ser Gln Pro Ser Ser Phe His Asp Phe Pro Asp Leu Gly Gln
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Glu Val Ala Leu Asn Ala Asn Thr Lys Asn Gln Lys Ile Arg Trp
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Lys Asn Glu Val Arg Ile His Ser Gly Ser Phe Gln Ser Gln Val
3635 3640 3645

Glu Leu Ser Asn Asp Gln Glu Lys Ala His Leu Asp Ile Ala Gly
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Ser Leu Glu Gly His Leu Arg Phe Leu Lys Asn Ile Ile Leu Pro
3665 3670 3675

Val Tyr Asp Lys Ser Leu Trp Asp Phe Leu Lys Leu Asp Val Thr
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Thr Ser Ile Gly Arg Arg Gln His Leu Arg Val Ser Thr Ala Phe
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Val Tyr Thr Lys Asn Pro Asn Gly Tyr Ser Phe Ser Ile Pro Val
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Lys Val Leu Ala Asp Lys Phe Ile Thr Pro Gly Leu Lys Leu Asn
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Asp Leu Asn Ser Val Leu Val Met Pro Thr Phe His Val Pro Phe
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Thr Asp Leu Gln Val Pro Ser Cys Lys Leu Asp Phe Arg Glu Ile
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Gln Ile Tyr Lys Lys Leu Arg Thr Ser Ser Phe Ala Leu Asn Leu
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Pro Thr Leu Pro Glu Val Lys Phe Pro Glu Val Asp Val Leu Thr
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Lys Tyr Ser Gln Pro Glu Asp Ser Leu Ile Pro Phe Phe Glu Ile
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Thr Val Pro Glu Ser Gln Leu Thr Val Ser Gln Phe Thr Leu Pro
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Lys Ser Val Ser Asp Gly Ile Ala Ala Leu Asp Leu Asn Ala Val
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Ala Asn Lys Ile Ala Asp Phe Glu Leu Pro Thr Ile Ile Val Pro
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Glu Gln Thr Ile Glu Ile Pro Ser Ile Lys Phe Ser Val Pro Ala
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Gly Ile Val Ile Pro Ser Phe Gln Ala Leu Thr Ala Arg Phe Glu

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Val Asp Ser Pro Val Tyr Asn Ala Thr Trp Ser Ala Ser Leu Lys
3890 3895 3900

Asn Lys Ala Asp Tyr Val Glu Thr Val Leu Asp Ser Thr Cys Ser
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Ser Thr Val Gln Phe Leu Glu Tyr Glu Leu Asn Val Leu Gly Thr
3920 3925 3930

His Lys Ile Glu Asp Gly Thr Leu Ala Ser Lys Thr Lys Gly Thr
3935 3940 39 45

Leu Ala His Arg Asp Phe Ser Ala Glu Tyr Glu Glu Asp Gly Lys
3950 3955 3960

Phe Glu Gly Leu Gln Glu Trp Glu Gly Lys Ala His Leu Asn Ile
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Lys Ser Pro Ala Phe Thr Asp Leu His Leu Arg Tyr Gln Lys Asp
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Lys Lys Gly Ile Ser Thr Ser Ala Ala Ser Pro Ala Val Gly Thr
3995 4000 4005

Val Gly Met Asp Met Asp Glu Asp Asp Asp Phe Ser Lys Trp Asn
4010 4015 4020

Phe Tyr Tyr Ser Pro Gln Ser Ser Pro Asp Lys Lys Leu Thr Ile
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Phe Lys Thr Glu Leu Arg Val Arg Glu Ser Asp Glu Glu Thr Gln
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Ile Lys Val Asn Trp Glu Glu Glu Ala Ala Ser Gly Leu Leu Thr
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Ser Leu Lys Asp Asn Val Pro Lys Ala Thr Gly Val Leu Tyr Asp
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Tyr Val Asn Lys Tyr His Trp Glu His Thr Gly Leu Thr Leu Arg
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Glu Val Ser Ser Lys Leu Arg Arg Asn Leu Gln Asn Asn Ala Glu
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Trp Val Tyr Gln Gly Ala Ile Arg Gln Ile Asp Asp Ile Asp Val

4115 4120 4125

Arg Phe Gln Lys Ala Ala Ser Gly Thr Thr Gly Thr Tyr Gln Glu

4130 4135 4140

Trp Lys Asp Lys Ala Gln Asn Leu Tyr Gln Glu Leu Leu Thr Gln

4145 4150 4155

Glu Gly Gln Ala Ser Phe Gln Gly Leu Lys Asp Asn Val Phe Asp

4160 4165 4170

Gly Leu Val Arg Val Thr Gln Lys Phe His Met Lys Val Lys His

4175 4180 4185

Leu Ile Asp Ser Leu Ile Asp Phe Leu Asn Phe Pro Arg Phe Gln

4190 4195 4200

Phe Pro Gly Lys Pro Gly Ile Tyr Thr Arg Glu Glu Leu Cys Thr

4205 4210 4215

Met Phe Ile Arg Glu Val Gly Thr Val Leu Ser Gln Val Tyr Ser

4220 4225 4230

Lys Val His Asn Gly Ser Glu Ile Leu Phe Ser Tyr Phe Gln Asp

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Leu Val Ile Thr Leu Pro Phe Glu Leu Arg Lys His Lys Leu Ile

4250 4255 4260

Asp Val Ile Ser Met Tyr Arg Glu Leu Leu Lys Asp Leu Ser Lys

4265 4270 4275

Glu Ala Gln Glu Val Phe Lys Ala Ile Gln Ser Leu Lys Thr Thr

4280 4285 4290

Glu Val Leu Arg Asn Leu Gln Asp Leu Leu Gln Phe Ile Phe Gln

4295 4300 4305

Leu Ile Glu Asp Asn Ile Lys Gln Leu Lys Glu Met Lys Phe Thr

4310 4315 4320

Tyr Leu Ile Asn Tyr Ile Gln Asp Glu Ile Asn Thr Ile Phe A sn

4325 4330 4335

Asp Tyr Ile Pro Tyr Val Phe Lys Leu Leu Lys Glu Asn Leu Cys
4340 4345 4350

Leu Asn Leu His Lys Phe Asn Glu Phe Ile Gln Asn Glu Leu Gln
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Glu Ala Ser Gln Glu Leu Gln Gln Ile His Gln Tyr Ile Met Ala
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Leu Arg Glu Glu Tyr Phe Asp Pro Ser Ile Val Gly Trp Thr Val
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Lys Tyr Tyr Glu Leu Glu Glu Lys Ile Val Ser Leu Ile Lys Asn
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Leu Leu Val Ala Leu Lys Asp Phe His Ser Glu Tyr Ile Val Ser
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Ala Ser Asn Phe Thr Ser Gln Leu Ser Ser Gln Val Glu Gln Phe
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Leu His Arg Asn Ile Gln Glu Tyr Leu Ser Ile Leu Thr Asp Pro
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Asp Gly Lys Gly Lys Glu Lys Ile Ala Glu Leu Ser Ala Thr Ala
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Gln Glu Ile Ile Lys Ser Gln Ala Ile Ala Thr Lys Lys Ile Ile
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Ser Asp Tyr His Gln Gln Phe Arg Tyr Lys Leu Gln Asp Phe Ser
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Asp Gln Leu Ser Asp Tyr Tyr Glu Lys Phe Ile Ala Glu Ser Lys
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Arg Leu Ile Asp Leu Ser Ile Gln Asn Tyr His Thr Phe Leu Ile
4520 4525 4530

Tyr Ile Thr Glu Leu Leu Lys Lys Leu Gln Ser Thr Thr Val Met
4535 4540 4545

Asn Pro Tyr Met Lys Leu Ala Pro Gly Glu Leu Thr Ile Ile Leu
4550 4555 4560

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<213> Homo sapiens

<400> 2

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Gln Ala Lys Val Glu Gln Ala Val Glu Thr Glu Pro Glu Pro Glu Leu
20 25 30

Arg Gln Gln Thr Glu Trp Gln Ser Gly Gln Arg Trp Glu Leu Ala Leu
35 40 45

Gly Arg Phe Trp Asp Tyr Leu Arg Trp Val Gln Thr Leu Ser Glu Gln
50 55 60

Val Gln Glu Glu Leu Leu Ser Ser Gln Val Thr Gln Glu Leu Arg Ala
65 70 75 80

Leu Met Asp Glu Thr Met Lys Glu Leu Lys Ala Tyr Lys Ser Glu Leu
85 90 95

Glu Glu Gln Leu Thr Pro Val Ala Glu Glu Thr Arg Ala Arg Leu Ser
100 105 110

Lys Glu Leu Gln Ala Ala Gln Ala Arg Leu Gly Ala Asp Met Glu Asp
115 120 125

Val Cys Gly Arg Leu Val Gln Tyr Arg Gly Glu Val Gln Ala Met Leu
130 135 140

Gly Gln Ser Thr Glu Glu Leu Arg Val Arg Leu Ala Ser His Leu Arg
145 150 155 160

Lys Leu Arg Lys Arg Leu Leu Arg Asp Ala Asp Asp Leu Gln Lys Arg
165 170 175

Leu Ala Val Tyr Gln Ala Gly Ala Arg Glu Gly Ala Glu Arg Gly Leu
180 185 190

Ser Ala Ile Arg Glu Arg Leu Gly Pro Leu Val Glu Gln Gly Arg Val
195 200 205

Arg Ala Ala Thr Val Gly Ser Leu Ala Gly Gln Pro Leu Gln Glu Arg
210 215 220

Ala Gln Ala Trp Gly Glu Arg Leu Arg Ala Arg Met Glu Glu Met Gly
225 230 235 240

Ser Arg Thr Arg Asp Arg Leu Asp Glu Val Lys Glu Gln Val Ala Glu
245 250 255

Val Arg Ala Lys Leu Glu Glu Gln Ala Gln Gln Ile Arg Leu Gln Ala
260 265 270

Glu Ala Phe Gln Ala Arg Leu Lys Ser Trp Phe Glu Pro Leu Val Glu
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Asp Met Gln Arg Gln Trp Ala Gly Leu Val Glu Lys Val Gln Ala Ala
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Val Gly Thr Ser Ala Ala Pro Val Pro Ser Asp Asn His
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